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Novel purification method of human immunoglobulin by using a thermo-responsive protein A

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ABSTRACT

We attempted to evaluate a novel purification method of immunoglobulins (IgGs) by using a mutant type of protein A. Although this mutant protein A binds to IgGs at 5 °C, the IgGs are released at 40 °C; hence, it was designated as thermo-responsive protein A (TRPA). We aimed to purify IgG1 from the culture supernatant of CHO cells producing AE6F4 human monoclonal IgG1. AE6F4 IgG1 was purified using only a TRPA-filled column and by modifying the temperature, without any exposure to acidic conditions. Furthermore, the purified AE6F4 IgG1 maintained the inherent binding affinity to antigen, while this property was lost in AE6F4 IgG1 purified using a conventional protein A (CPA) column possibly because of product aggregation and fragmentation. These data suggest that IgG is sensitive to acid treatment; however, it can be highly purified with retention of high affinity by using a TRPA column. Further, this purification method can be used on an industrial scale for the purification of antibody drugs.

1. Introduction

Antibodies are biologically active agents that regulate the immune system. Recently, the availability of antibodies for use in drugs and diagnosis, and as a purification tool for antigens has greatly increased [1]. Immunoglobulins (Igs) can be purified from the blood of immunized animal, ascites of mice injected with hybridomas, and culture supernatant of cells producing antibodies. However, these fluids contain various impurities as well as the target Igs. Therefore, although time-consuming and troublesome, the purification of Igs from these fluids is very important.

For IgG purification, protein A-coupled affinity chromatography is widely used because protein A can specifically bind to the Fc region of IgG with high affinity. However, during the purification process, the IgGs are loaded onto protein A column under neutral condition (around pH 7) and are then eluted under acidic conditions (pH 3–4). Thus, it is very difficult to purify acid-sensitive IgGs, as the acidic conditions may result in the denaturation and aggregation of IgGs and reduced purification efficiency. In particular, when purifying human monoclonal IgGs that are used as antibody drugs, prevention of aggregation and inactivation of IgGs are the most important issues [2–4].

To overcome these difficulties, we propose the use of a mutant protein A (thermo-responsive protein A [TRPA]) [5–8]. TRPA can be obtained as previously described [5]. Briefly, mutations were introduced into the IgG-binding domain of Protein A to decrease the hydrophobicity of the side chains buried inside the domain. Such mutations were designed to modulate the equilibrium between the denatured state and native state so that the domain has the native structure at low temperature, e.g., below 10 °C, while it denatures at elevated, yet biologically acceptable temperatures, e.g., approximately 40 °C. In the present study, IgG was bound to TRPA at 5 °C, and bound IgG was released at 40 °C. We then evaluated the efficiency of mutant protein A in the purification of native structured IgG and the effects on its function.

2. Experimental methods

2.1. Materials and reagents

TRPA resin is made up of mutant protein A-coated porous beads made of cross-linked polyvinyl alcohol (average particle size: 60 μm; Nomadic Bio Science, Okayama, Japan). MabSelect (GE

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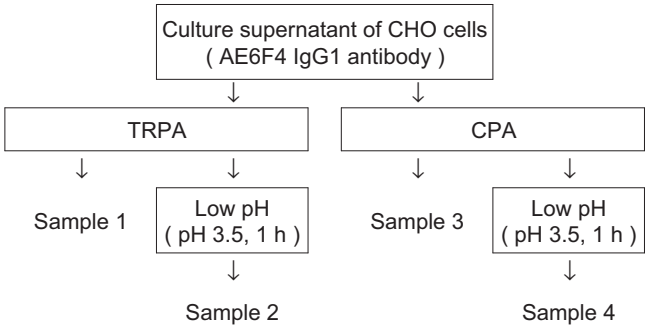

Fig. 1. Purification process of human AE6F4 IgG1. Culture supernatant of CHO cells producing human AE6F4 IgG1 was harvested and membrane filtered using BioOptimal MF-SL. The supernatant was applied to the TRPA and CPA columns, eluted, and designated as Samples 1 and 2, respectively. Furthermore, AE6F4 human IgG1 purified using TRPA and CPA was placed in acidic conditions for 1 h, neutralized to pH 5.0, and designated as Samples 3 and 4, respectively.

Healthcare Japan, Tokyo, Japan), a protein A-coated porous agarose bead (average particle size: 85 μm) was used as the conventional protein A (CPA) resin. Human polyclonal antibody (IgG1, Benesis, Osaka), human IgG1 (Sigma, St. Louis, MO), human IgG2 (Merck Millipore, Billerica, MA), human IgG4 (Abcam, Cambridge, MA), mouse IgG1 (Sigma), mouse IgG2 (Sigma), goat IgG1 (Sigma), chicken IgY (Wako Pure Chemical, Osaka, Japan), and rabbit IgG1 (Sigma) were used as control IgGs. AE6F4 cells were used as the human IgG1 producer cell line [9]. BioOptimal MF-SL (Asahi Kasei Medical, Tokyo, Japan) was used to fine-filter the culture medium. Cytokeratin 8 was purchased from Progen (Heidelberg, Germany).

2.2. Purification of IgG using protein A resin

The Tricorn 5/100 column (GE Healthcare) was filled using a 50% slurry of TRPA or CPA, which was connected to an $AKTA_{FPLC}$ (GE Healthcare). The temperature of the column filled with TRPA was controlled by a temperature-regulated water bath. When purifying IgG using the column filled with TRPA resin (TRPA column), we applied the filtered culture supernatant containing AE6F4 IgG1 to the TRPA column at a flow rate of 0.4 mL/min at 2 °C. The TRPA column was washed with 10 mL of buffer 1 (20 mM phosphate buffer, 150 mM NaCl, pH 8.0), then kept in a water bath at 40 °C for 10 min. Following this, AE6F4 IgG1 was eluted using buffer 1. The eluted IgG was designated as Sample 1 (Fig. 1, Table 1).

When purifying IgG using the column filled with CPA resin (CPA column), we applied the filtered culture supernatant containing AE6F4 IgG1 to the CPA column at room temperature at a flow rate of 0.4 mL/min. The CPA column was washed with 10 mL of buffer 1. Bound AE6F4 IgG1 was eluted using acidic buffer (50 mM citrate buffer, pH 3.0, 300 mM NaCl), and neutralized to pH 5.0 with 5% volume of 1 M Tris–HCl buffer, pH 8.0. The eluate was at the low pH condition prior to neutralization of the protein approximately 6 min. The eluted antibody was designated as Sample 3 (Fig. 1, Table 1).

2.3. Low-pH treatment

The pH of the TRPA eluate was adjusted to 3.5 by adding acetic acid, incubated for 1 h at room temperature, and neutralized to pH 5.0 by adding 1 M Tris–HCl (pH 8.0); this sample was designated as Sample 2. The pH of the CPA eluate was adjusted to 3.5 by adding acetic acid, incubated for 1 h at room temperature, and neutralized to pH 5.0 by adding 1 M Tris–HCl (pH 8.0); this sample was designated as Sample 4 (Fig. 1, Table 1).

2.4. Isolation of monomer AE6F4 IgG1 by size-exclusion column chromatography

Eluates treated as above (Samples 1–4) containing 5–10 mg of AE6F4 IgG1 were applied to size-exclusion column chromatography (SEC; HiLoad 16/60 Superdex 200 prep grade, GE Healthcare) using sodium phosphate buffer (50 mM phosphate, 150 mM NaCl, pH 7.2) at a flow rate of 1.0 mL/min. The elution profile was monitored at an absorbance of 280 nm, and the main peak fraction consisting of the monomer AE6F4 IgG1 was collected.

2.5. Analysis of affinity between AE6F4 IgG1 and cytokeratin 8 using a surface plasmon resonance (SPR) biosensor

Real-time analysis of the interaction between AE6F4 IgG1 and cytokeratin 8 was studied using SPR measurements (Biacore J, GE Healthcare). Cytokeratin 8 was coupled onto a sensor chip CM5 (GE Healthcare) using an Amino Coupling kit (GE Healthcare). For binding experiments, the Biacore J was operated at 25 °C; filtered (0.22 μm) and degassed HBS-EP running buffer (GE Healthcare) was used for all assays. The contact time of analyte and ligand was limited to 120 s. For SPR biosensor analysis, 70 μL of solution containing AE6F4 IgG1 was injected at a flow rate of 30 μL/min. Each experiment was performed at least in triplicate. The K_D value was calculated using BIAEvaluation in the Biacore J program.

3. Results and discussion

3.1. The effect of temperature on the binding of IgG to TRPA

Human polyclonal IgG1 was dissolved at a concentration of 5 mg/mL in sodium phosphate buffer (neutral buffer, 20 mM phosphate, 150 mM NaCl, pH 8.0). This IgG1 solution was applied to the temperature-controlled TRPA column from 2 °C to 40 °C. The column was washed using neutral buffer, and the bound IgG1 was eluted by raising the column temperature to 40 °C.

The temperature-dependent IgG1 binding capacity to the TRPA column is shown in Fig. 2. The concentration of IgG1 in the

Table 1
Purification process using TRPA and CPA.

	Sample 1	Sample 2	Sample 3	Sample 4
Protein A type	TRPA	TRPA	CPA	CPA
Elution buffer	20 mM phosphate + 150 mM NaCl (pH 8.0)	20 mM phosphate + 150 mM NaCl (pH 8.0)	50 mM citrate + 300 mM NaCl (pH 3.0)	50 mM citrate + 300 mM NaCl (pH 3.0)
Elution temperature	40 °C	40 °C	25 °C	25 °C
Neutralization buffer	–	–	1 M Tris–HCl (pH 8.0)	1 M Tris–HCl (pH 8.0)
pH after neutralization	–	–	pH 5.0	pH 5.0
Low pH buffer	–	Acetic acid	–	Acetic acid
Low pH incubation	–	pH 3.5, 1 h	–	pH 3.5, 1 h
Neutralization buffer	–	1 M Tris–HCl (pH 8.0)		1 M Tris–HCl (pH 8.0)
pH after neutralizaiton	–	pH 5.0	–	pH 5.0

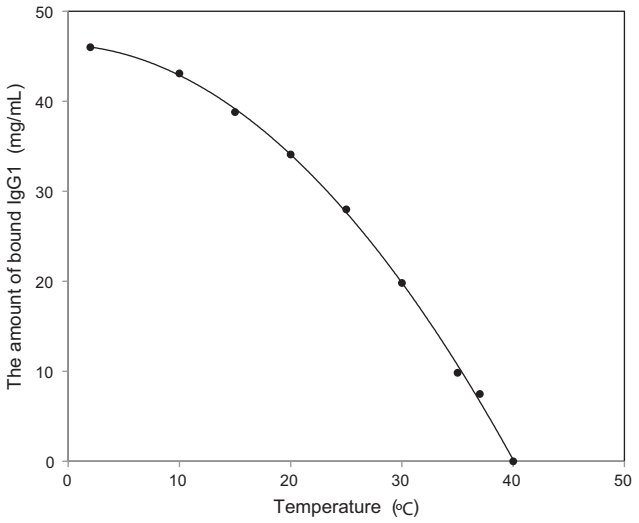

Fig. 2. Effect of column temperature on the binding of IgG1 to TRPA resin. Human polyclonal IgG1 (5 mg/mL in 20 mM phosphate and 150 mM NaCl, pH 8.0) was applied to the temperature-controlled TRPA column at 2–40 °C. After the column was washed using the buffer, bound IgG was eluted by raising the temperature to 40 °C. The concentration of IgG1 in the eluate was determined by the absorbance at 280 nm and the molar extinction coefficient of IgG.

eluate was determined by the absorbance at 280 nm and the molar extinction coefficient of IgG (1.35 L/[mol cm]). The maximum binding capacity of human IgG1 was estimated to be 46 mg/mL resin at 2 °C. As the temperature increased, the IgG1 binding capacity decreased. At 40 °C, the TRPA resin was unable to bind to IgG1. These results indicate that TRPA, a mutant type of protein A, binds strongly to human IgG1 at low temperature, but does not bind at temperatures higher than 40 °C under neutral buffering conditions. In addition, this temperature-dependent binding to the TRPA column was observed for human IgG2 and human IgG4, suggesting that this technology can be widely used for other subclasses of IgGs (Fig. S1).

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Furthermore, after binding at 2 °C and elution at 40 °C, the TRPA column can be cleaned by treatment with 8 M urea. Human IgG1 was then loaded onto this restored TRPA column at 2 °C, and captured IgG1 was eluted at 40 °C. The amount of eluted IgG1 was almost the same as that of applied IgG1. This cleaning procedure was performed for 10 cycles, and the adbsorbent cleanability was confirmed. These results indicate that the TRPA column is resistant to the cleanup procedure by using 8 M urea and tolerant to repeated use for industrial production processes.

Table 2
Static binding capacity of IgG to protein A.

Species	Subclass	Static binding capacity of IgG (mg/mL chromatography adsorbent)	
		Conventional protein A resin (25 °C)	Thermo-responsive protein A resin (10 °C)
Mouse	IgG1	3.6	0.2
	IgG2	4.7	0.6
Goat	IgG1	12.4	3.4
Chicken	IgY	0.4	0.7
Rabbit	IgG1	13.4	10.4
Human	IgG1	15.1	10.7
	IgG2	15.4	5.7
	IgG4	10.9	10.8

3.2. Binding affinity of TRPA to various IgGs

We assessed the static binding capacity of TRPA with various IgG subtypes derived from various animals. All IgGs were dissolved in neutral buffer at a concentration of 0.5 mg/mL. TRPA (0.01 mL) was added to the IgG solution (1.0 mL) and incubated at 10 °C for 1 h. After this, the concentration of IgG in the supernatant was determined by the absorbance at 280 nm, and the static binding capacity of TRPA was calculated.

CPA (0.01 mL) was added to the antibody solution (1.0 mL) and incubated at room temperature for 1 h. The pH was then adjusted to 3.0 by adding acetic acid, and the supernatant was recovered. The static binding capacity of CPA was calculated as mentioned previously. The static binding capacities of TRPA and CPA to various IgGs are shown in Table 2. These data demonstrated that TRPA was unable to bind IgGs derived from mice, suggesting that the mutations introduced into protein A affect the binding of TRPA to mouse-derived IgG. However, other IgGs bound similarly to TRPA and CPA.

3.3. Purification of human IgG1

3.3.1. Purification of human IgG1 using TRPA and CPA

Culture supernatant containing AE6F4 IgG1 was filtered using the precise membrane (BioOptimal MF-SL, Asahi Kasei Medical). The supernatant was applied to TRPA and CPA columns, and the captured AE6F4 IgG1 was eluted as described above. Elution profiles when using TRPA and CPA columns are shown in Fig. 3A and B, respectively. We applied 16.1 mg/mL (TRPA) and 27.1 mg/mL (CPA) of IgG1 to the respective columns, which corresponded to about 80% of the dynamic binding capacity for IgG1 at 10% breakthrough (20 mg/mL IgG1 for TRPA and 35 mg/mL for CPA). The recovery rate of AE6F4 IgG1 was 88% when using the TRPA column and was 30% when using the CPA column. These results indicate that elution at pH 3.0 is not an optimal recovery condition for AE6F4 IgG1,

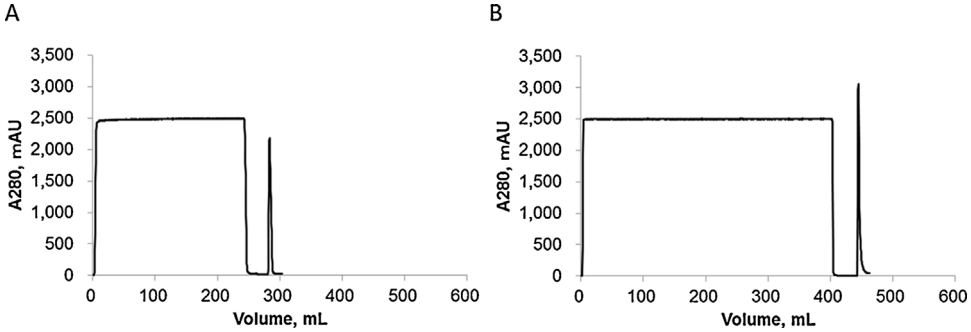

Fig. 3. Elution profile of AE6F4 IgG1. Culture supernatant was applied to the TRPA and CPA columns, and the captured AE6F4 IgG1 was eluted as described in the Experimental section. The elution profiles by using the (A) TRPA and (B) CPA columns are shown.

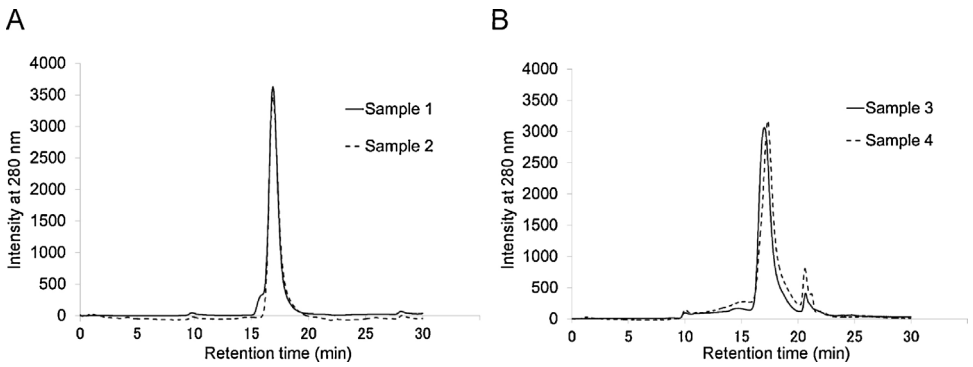

Fig. 4. Size-exclusion column chromatography (SEC) analysis of AE6F4 IgG1. Elution profiles of SEC for AE6F4 IgG1 purified by the (A) TRPA and (B) CPA column before (solid lines) and after (dotted lines) exposure to acidic conditions for 1 h at room temperature.

suggesting the need for optimizing the elution conditions for each IgG when using the CPA column.

3.3.2. Molecular species of AE6F4 IgG1 analyzed by size-exclusion chromatography

We next analyzed the molecular species of AE6F4 IgG1 purified by using the TRPA and CPA columns by SEC. AE6F4 IgG1 purified by the TRPA column showed almost a single monomer peak (Sample 1, Fig. 4A) and a small shoulder peak. On the other hand, AE6F4 IgG1 purified by the CPA column contained several molecular species, possibly corresponding to aggregated IgG1 with a high molecular weight and degraded IgG1 with a low molecular weight as well as monomer (Sample 3, Fig. 4B). The formation of these molecules could be attributed to the acidic elution buffer that was used.

Next, AE6F4 IgG1 purified using TRPA and CPA was placed in an acidic condition for 1 h, and then applied to SEC (Samples 2 and 4, respectively). Results showed that these IgG1s showed different elution profiles after the low pH treatment. AE6F4 IgG1 purified using the TRPA column and subsequent low pH treatment was eluted as a single peak, devoid of the shoulder peak adjacent to the main peak (Fig. 4A, dotted line). Retention time of the main peak remained unchanged upon the low pH treatment. The disappearance of the shoulder peak might be caused by the dissociation of aggregated antibody by the low pH treatment. On the other hand, AE6F4 IgG1 purified by using the CPA column showed a complex elution profile after the low pH treatment (Fig. 4B, dotted line). The amount of high and low molecular weight species increased and the retention time of the main peak increased upon low pH treatment. These results suggest that AE6F4 IgG1 purified using the CPA column is susceptible to aggregation and degradation possibly because it went through the acid elution at pH 3.0. The structure of AE6F4 IgG1 could be more fragile against acid treatment. Aggregation of AE6F4 IgG1 formed possibly by inappropriate interactions would dissociate by transient exposure to pH 3.5 (Fig. 4A), but transient exposure to pH 3.0 would denature AE6F4 IgG1 and increase the aggregation (Fig. S2). Taken together, these data suggest that when purifying IgGs with an acid-sensitive structure, purification using the TRPA column is more suitable than purification using the CPA column; sensitive and unstable IgGs can be purified using the TRPA column.

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3.3.3. Binding affinity of AE6F4 IgG1 to cytokeratin 8

The affinities of AE6F4 IgG1 purified by using the TRPA and CPA columns (Samples 1 and 3) and those treated at low pH (Samples 2 and 4) with antigen (cytokeratin 8) were determined using BiacoreJ (Table 3). First, results show that the K_D value of AE6F4 IgG1 purified using the TRPA column (Sample 1) for cytokeratin 8 was lower than that of AE6F4 IgG1 purified using the CPA column (Sample 3), indicating that the binding affinity of AE6F4 IgG1 purified using the TRPA column was more than 4-fold higher than that of AE6F4 IgG1 purified using the CPA column. These results suggest that AE6F4 IgG1 purified using the TRPA column (Sample 1) maintains the native structure and binding affinity against antigen possibly because AE6F4 IgG1 was eluted from the TRPA column under mild conditions (neutral buffer, pH 8.0) without any exposure to acidic conditions. On the other hand, AE6F4 IgG1 purified using the CPA column (Sample 3) is likely partially denatured, which could reduce the binding affinity for antigen possibly due to exposure to acidic conditions (pH 3.0).

Table 3
Binding affinity of IgG1 purified using TRPA and CPA.

	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (M)	Relative binding affinity
Sample 1	1.06E+04	2.77E−03	2.61E−07	100
Sample 2	5.18E+03	1.19E−01	2.30E−05	1
Sample 3	1.59E+04	1.74E−02	1.09E−06	24
Sample 4	5.07E+03	5.09E−02	1.00E−05	3

Furthermore, both binding affinities of AE6F4 IgG1 purified using TRPA and CPA against antigen were greatly decreased upon acid treatment (Samples 2 and 4). These results suggest that AE6F4 IgG1 is sensitive to acid treatment, and the structure of AE6F4 IgG1 is easily denatured upon acid treatment, resulting in decreased binding affinity to antigen. Thus, the TRPA column is best suited for the purification of acid-sensitive IgGs, and it may be applicable for the industrial-scale purification of IgGs that could denature upon acid elution using the CPA column.

4. Conclusion

IgG1 was purified using the TRPA column by only modulating the temperature, with no exposure to acidic conditions. When using the TRPA column, IgG1 was purified mainly as a monomer. On the other hand, IgG1 purified using the CPA column contained various molecular species such as aggregated and degraded IgG1. Furthermore, IgG1 purified using TRPA maintained the inherent binding affinity to antigen, while this ability was decreased in IgG1 purified by CPA. These results suggest that this IgG1 is sensitive to acid treatment, but can be highly purified with retention of high-affinity binding using the TRPA column, even for industrial scale purification of antibody drugs.

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